Appendix B

CD18-Mediated Neutrophil Recruitment Contributes to the Pathogenesis of Reperfused but Not Nonreperfused Stroke

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Background and Purpose—Neutrophil (PMN) recruitment mediated by increased expression of intercellular adhesion molecule-1 expression (ICAM-1, CD54) in the cerebral microvasculature contributes to the pathogenesis of tissue injury in stroke. However, studies using blocking antibodies against the common β_2-integrin subunit on the PMN, the counterligand for ICAM-1 (CD18), have demonstrated equivocal efficacy. The current study tested the hypothesis that mice deficient in CD18 would be protected in the setting of reperfused but not nonreperfused stroke.

Methods—Two groups of mice were studied, those whose PMNs could express CD18 (CD18 +/+) and those mice hypomorphic for the CD-18 gene (CD18 −/−). PMNs obtained from CD18 −/− or CD18 +/+ mice were fluorescently labeled and tested for binding to murine brain endothelial monolayers. Using a murine model of focal cerebral ischemia in which an occluding suture placed in the middle cerebral artery (MCA) is removed after 45 minutes (transient ischemia, reperfused stroke) or left in place (permanent ischemia, nonreperfused stroke), cerebral infarct volumes (% ipsilateral hemisphere by TTC staining), cerebral blood flow (CBF, % contralateral hemisphere by laser-Doppler flowmetry), and survival (%) were examined 24 hours after the initial ischemic event. Adoptive transfer studies used ^{111}In-labeled PMNs (from either CD18 +/+ or CD18 −/− mice) to examine the relative accumulation of PMNs in the ischemic region.

Results—PMNs obtained from CD18 −/− mice exhibit reduced adhesivity (compared with CD18 +/+ PMNs) for both quiescent and cytokine-activated endothelial monolayers. CD18 −/− mice (n=14) subjected to transient focal cerebral ischemia demonstrated a 53% decrease in infarct volumes versus CD18 +/+ mice (n=26, $P<0.05$), improved penumbral CBF at 24 hours (1.8-fold, $P=0.02$), and a 3.7-fold decrease in mortality ($P=0.02$). However, when CD18 −/− mice (n=12) were subjected to permanent focal cerebral ischemia, no differences were noted in infarct volume, mortality, or CBF versus similarly treated CD18 +/+ mice (n=10). There was a greater accumulation of CD18 +/+ PMNs in the ischemic zone of CD18 +/+ animals than CD18 −/− animals subjected to reperfused stroke (82% increase, $P=0.02$), although there was no difference between groups when subjected to permanent MCA occlusion.

Conclusions—Deficiency for the CD18 gene confers cerebral protection in a murine model of reperfused stroke, but this benefit does not extend to CD18-deficient animals subjected to permanent MCA occlusion. These data suggest that anti-PMN strategies should be targeted to reperfused stroke and may perhaps be used in conjunction with thrombolytic therapy that establishes reperfusion. (***Stroke.*** **1999;30:1110-1117.)**

Key Words: antigens, CD18 ■ endothelium ■ leukocytes ■ reperfusion ■ stroke, experimental ■ mice

When a patient suffers an ischemic stroke, there are relatively few established therapies that can be initiated immediately and have been proved to improve outcome. Initial studies of embolic stroke in rabbits showed that lysing thrombi could reduce neurological damage.[1] Recent clinical trials have shown early reperfusion with tPA[1] to be beneficial in terms of reducing long-term morbidity.[2,3] Local intra-arterial thrombolysis with recombinant prourokinase has shown initial promise in small, early trials if given within a 4-hour window,[4,5] although, as with all thrombolytic agents, intracerebral hemorrhage remains a concern. Streptokinase

See Editorial Comment, page 1116

increases mortality in ischemic stroke.[6] Although timely reperfusion can rescue jeopardized brain tissue, there are theoretical risks attendant with reperfusion in addition to the recognized risk of hemorrhagic conversion. In the highly vulnerable reperfusion period, recruited neutrophils release a veritable firestorm of reactive oxygen intermediates, corrosive acids, and cytolytic enzymes.[7] Recent evidence suggests that reperfusion may be deleterious in certain circumstances, leading to an increase in myocardial[8] or cerebral[9] infarct size.

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Stroke **is available at http://www.strokeaha.org**

Exhibit A

In one report of reversible unilateral middle cerebral artery/common carotid artery occlusion in the rat, up to 72% of the ischemic damage to the brain was a consequence of reperfusion injury.[9] However, because of the recent recognition of the primacy of timely reperfusion in cerebral tissue salvage in the face of an ischemic event, it becomes imperative to understand mechanisms through which potential toxicities of the reperfusion milieu can be diminished.

There are recent data showing that P-selectin[10] and ICAM-1,[11] potent neutrophil adhesion receptors whose expression is increased on the surface of postischemic cerebral endothelial cells, participate in the pathogenesis of both neutrophil recruitment and cerebral tissue injury in reperfused stroke. However, there is limited data to indicate whether inhibiting P-selectin– or ICAM-1–mediated leukocyte recruitment may be beneficial in strokes that fail to reperfuse. Patients usually present after the safety window for thrombolytic intervention, and it is therefore important to understand whether mechanisms that protect in reperfused stroke might enable salvage of jeopardized penumbral tissue in strokes that do not reperfuse. In a recent clinical trial in which a blocking antibody to human ICAM-1 was administered within 6 hours after stroke, no therapeutic benefit was observed and the trial was aborted (Reference 12 and Stephen Polmar, oral communication). The reasons for this clinical failure are unclear, but one reason this trial may have failed to demonstrate a beneficial effect of anti-leukocyte adhesion therapy is that the majority of the patients did not reperfuse (Stephen Polmar, personal communication).

ICAM-1 mediates firm neutrophil arrest to activated endothelial cells by binding to β_2-integrins, heterodimeric adhesion receptor glycoproteins expressed on the neutrophil surface. CD18 is the common β_2-subunit located on the neutrophil surface (common to both LFA-1 [CD11a/CD18] and Mac-1 [CD11b/CD18]) and is responsible for ICAM-1–mediated leukocyte adhesion to endothelial cells.[13] Its role in the pathogenesis of ischemic cerebral damage has not been clearly defined. In animal models of cerebral or spinal stroke, administration of blocking antibody to CD18 either improved outcome[14,15] or had no effect on outcome.[16] Based on the recent identification of the importance of ICAM-1 in the pathogenesis of reperfused stroke, we hypothesized that mice deficient in CD18 would be protected from cerebral ischemia; furthermore, given the negative data in the EnlimoMab trial, we hypothesized that the beneficial effects of CD18 deficiency would be most apparent in a model of reperfused (compared with nonreperfused) stroke. To test these hypotheses, deletionally mutant mice (hypomorphic for CD18[17]) were used to study the effects of focal cerebral ischemia with or without reperfusion.

Materials and Methods

Animals

Adult male C57Bl/6J mice homozygous null for the Itgb2 mutation (C57Bl/6J-Itgb2tm1Bay, derived from the tenth generation of backcrossing with C57Bl/6J mice, and therefore comprised of 99.8% of the C57Bl/6J genotype)[17] and controls (C57Bl/6J) were obtained from The Jackson Laboratory (Bar Harbor, Maine). The C57Bl/6J-Itgb2tm1Bay mice are hypomorphic homozygotes for the CD18 allele[17]; throughout this paper, these mice will be referred to as CD18 −/−; C57Bl/6J mice will be designated CD18 +/+. All mice were between 7 and 10 weeks of age and weighed between 23 and 32 grams at the time of surgery. The latter part of the C57Bl/6J-Itgb2tm designation is one of nomenclature: Itgb2=integrin beta 2; tm1=targeted mutation 1; Bay=Baylor, the institution where the mice were created.

Middle Cerebral Artery Occlusion

Mice were subjected to transient and permanent middle cerebral artery (MCA) occlusion through procedures that were approved by the university's Animal Care and Use Committee and are similar to those which have been recently reported in detail.[18] Briefly, mice were anesthetized with an intraperitoneal injection of 0.3 mL of a combination of ketamine (10 mg/mL) and xylazine (0.5 mg/mL). A rectal temperature probe (Yellow Springs Instruments) connected via thermocouple to an infrared heat source was used to maintain a core temperature of 36°C to 38°C during the perioperative period. With the animal in the supine position, a 1-cm midline neck incision was made and the right common carotid artery (CCA), internal carotid artery (ICA), and external carotid artery (ECA) were identified and exposed. With the aid of an operating microscope (10-40× zoom, Leica), the CCA was isolated with 4–0 silk sutures, and proximal and distal control of blood flow was obtained by applying gentle traction to the silk sutures, effectively occluding the CCA. After cautery and transection of the ECA, an arteriotomy was made on the proximal stump, and a heat-blunted nylon suture was introduced into the proximal lumen. The suture tip was then advanced up the ICA to the origin of the right MCA, after which the arteriotomy site was cauterized, and traction on the CCA was released. Total carotid occlusion time was <2 minutes in all cases. Transcranial measurements of relative cerebral blood flow (CBF, as described below) were used to confirm occlusion of the MCA. Occlusion was considered to be technically adequate if ≥50% reduction in relative CBF was observed immediately after placement of the intraluminal occluding suture.

In animals undergoing permanent MCA occlusion, the intraluminal MCA suture was left in place, all skin incisions were closed with surgical staples, and the animal was placed in an incubator to maintain core temperature at a constant 37°C for 90 minutes during the animal's recovery from surgery and anesthesia. Animals undergoing transient MCA occlusion were maintained at a core temperature of 37°C with the occluding catheter in place. After 45 minutes of ischemia, CBF was again assessed, the catheter was withdrawn, and the arteriotomy site was cauterized. Reestablishment of blood flow to the MCA distribution was ascertained by laser Doppler flowmetry. All incisions were subsequently closed with surgical staples; the animals were maintained at 37°C and allowed to recover from the effects of anesthesia in an incubator for 90 minutes. All animals were then returned to their respective cages and given free access to food and water.

Quantitation of Cerebral Infarct Volumes

On postoperative day 1 (24 hours postoperatively), the mice were anesthetized, relative CBF measurements were again evaluated, and the animals were sacrificed by rapid decapitation. One mm thick coronal brains sections were cut using a mouse brain matrix (Activation Systems, Inc), and sections were immersed in 2% 2,3,5-triphenyltetrazolium chloride (TTC; Sigma Chemical Co) in 0.9% saline solution, as described.[19] Serial sections were then photographed adjacent to a 1-cm reference bar, and areas of infarction were traced by a member of the team blinded to the experimental conditions. Through the use of computerized imaging software (NIH Image), total volume of infarcted tissue was expressed as a percentage of total right hemispheric volume. Although this method for calculating infarct volumes may overestimate infarct volumes because of swelling of the ipsilateral brain, especially during the early period (<24 hours) under study, all animals were treated identically and all infarct volumes calculated in the same manner.

Evaluation of CBF

CBF evaluations were performed using a 0.7-mm straight laser Doppler flowmeter (Perimed, Inc). After reflecting the skin overlying the translucent calvarium in anesthetized animals, both cerebral convexities were visualized. By placing the probe over the right and left hemispheres perpendicular to the calvarial surface 2 mm posterior to the bregma, 6 mm lateral to the midline (designated "core"), and 3 mm lateral to the midline (designated "penumbra"), relative blood flow measurements were obtained. Measurements were thus obtained using these rigid coordinates so that they would be objective and reproducible, but due to slight variations in infarct regions, they may not represent true core or penumbral regions as defined by other techniques. Measurements were obtained on all animals before MCA occlusion, immediately after introduction of the intraluminal suture, and immediately before sacrifice. In instances where animals were subjected to transient focal cerebral ischemia, additional measurements were obtained before removal of the occluding intraluminal suture and again immediately after MCA perfusion was reestablished. Data are expressed as the ratio of the Doppler signal intensity of the ischemic compared with that of the nonischemic hemisphere.

Neurological Examination

Twenty-four hours after MCA occlusion and reperfusion, before being given anesthesia, mice were examined for neurological deficit by use of a 4-tiered grading system:[18] A score of 1 was given if the animal demonstrated normal spontaneous movements; a score of 2 was given if the animal was noted to be turning to the right (clockwise circles) when viewed from above; a score of 3 was given if the animal was observed to spin longitudinally (clockwise when viewed from the tail); and a score of 4 was given if the animal was crouched on all fours, unresponsive to noxious stimuli. This scoring system has been previously described in mice.[18]

Preparation and Administration of ^{111}Indium Oxine-Labeled Murine Neutrophils (PMNs)

Homozygous C57Bl/6J-Itgb2tm1Bay and wild type C57Bl/6J mice were anesthetized, and 0.5 to 1.0 mL of blood was withdrawn from each mouse by percutaneous intracardiac puncture with a 22-gauge needle and transferred to a sterile test tube with 0.1 mL sodium citrate at room temperature. Blood was then diluted 1:1 with PBS, transferred to a 15-mL conical tube containing Ficoll-Hypaque (Pharmacia LKB Technology), and centrifuged at 1800 rpm for 20 minutes at room temperature. The buffy coat was then gently transferred to a second conical tube and centrifuged at 1400 rpm for 15 minutes at 4°C. The supernatant was aspirated, and red blood cells were subjected to hypotonic lysis; the remaining cells were then resuspended in PBS. The sample was centrifuged at 1200 rpm for 12 minutes at 4°C, the supernatant was decanted, and the hypotonic lysis step was repeated until the specimen was free of erythrocytes. The leukocytes were then resuspended in PBS to a count of 5 to 7.5×106 cells/mm^3 and incubated at 37°C for 15 minutes with 100 μCi ^{111}In-oxine (Amersham Mediphysics) The neutrophils were then centrifuged at 1800 rpm for 5 minutes and washed 3 times with PBS at 37°C. The neutrophils were then resuspended to a final concentration of 1.0×106 cells/mL. Final counts were adjusted to ≈3×106 cpm/0.3 mL by admixture with physiological saline; this was given via penile vein injection to anesthetized animals before surgery. Animals were killed at 24 hours after occlusion, and ^{111}In-PMN deposition was quantified as counts per minute per gram of tissue and reported as a ratio of PMN accumulation in the ischemic versus the nonischemic hemisphere.

Cerebrovascular Anatomy

In order to evaluate differences in cerebrovascular anatomy between CD18 −/− and CD18 +/+ animals, thoracotomy was performed on anesthetized animals and a 0.1-mL injection of India ink/carbon black/methanol/physiological saline (1:1:1:1, v:v:v:v) was administered by cardiac puncture. Mice were subsequently decapitated, and the brains were harvested and immersed in 10% formalin at 4°C for 48 hours. Specimens were then photographed to define the anatomy of the circle of Willis and its major branches.

Preparation of Fluorescently Labeled Murine Neutrophils

Fluorescently-labeled neutrophils were obtained from pooled citrated blood obtained from cardiac puncture of either CD18+/+ or CD18 −/− mice (10 animals from each group), which were prepared as described above for the radiolabeled neutrophils, except that the labeling protocol differed. Detection of neutrophil adhesion was performed using a fluorometric assay in which fluorescently-labeled neutrophils which adhere to an endothelial cell monolayer are detected based on the application of an excitatory wavelength (485 nm) and detection of the emission wavelength (530 nm).[20] The fluorescent marker, calcein acetoxymethyl ester, is a membrane permeable dye which fluoresces when the acetoxymethyl group is cleaved by cellular esterases in living cells. The calcein-am was prepared by combining 3 μL of calcein-am in 3 μL of pluronic F-127 (both supplied by Molecular Probes; the calcein-AM was first brought to 10 mmol/L in anhydrous dimethylsulfoxide and frozen in aliquots prior to use), and adding this to 60 mL of heat-inactivated calf serum and 2.9 mL of PBS. Following the initial preparatory steps, neutrophils were washed twice with PBS, and added to the tube containing the calcein-am (approximately 3×10^6 cells/3 mL dye) and incubated for 40 minutes at room temperature in the dark with gentle agitation. Cells were then collected by centrifugation, washed 3 times in PBS, and then resuspended in RPMI.

Binding Assay

Murine brain endothelial cells (a generous gift of Dr R. Auerbach,[21] University of Wisconsin), which we have used for other experiments in our laboratory,[22] were grown to confluence on a 24-well plate. Cells were either incubated in medium alone or activated with recombinant murine IL-1 β (2.5 ng/mL, R&D Systems, Minneapolis, MN) for 20 hours, after which medium from the plate was removed and the labeled neutrophil suspension added (3×10^5 cells/well). Cocultures were incubated together for 1 hour at 37°C in a cell culture incubator, after which monolayers were washed 4 times with PBS. Fluorescent intensity was read using a Cytofluor Series 4000 (Perkin-Elmer). Five wells were used for each of the 4 conditions tested (CD18 +/+, quiescent endothelial cells; CD18 −/−, quiescent endothelial cells; CD18 +/+, activated endothelial cells; CD18 −/−, activated endothelial cells). Data are expressed as mean±SEM fluorescent intensity.

Data Analysis

CBF, infarct volumes and ^{111}In-PMN deposition were compared using the Student's t test for unpaired variables. Neurological deficit scores were compared using the Mann-Whitney U test. Survival analysis was tested using contingency analysis with the Chi square statistic. Values are expressed as the mean±SEM, with a $P<0.05$ considered statistically significant.

Results

In order to determine the specific role of CD18 in the pathogenesis of tissue injury in stroke, CD18 −/− mice were used. Because there can be variations in cerebrovascular anatomy between strains of mice which can affect the severity of cerebral ischemic tissue damage,[23] initial experiments were performed to ascertain that the CD18 −/− mice did not have grossly detectable variations in cerebrovascular anatomy. For these experiments, an antemortem intravascular injection of India Ink was used to define the Circle of Willis and its principal branches. These experiments showed, from a gross morphologic perspective, that for both CD18 +/+ and CD18 −/− mice, there is a complete Circle of Willis with no evidence of aberrant communicating vessels between the

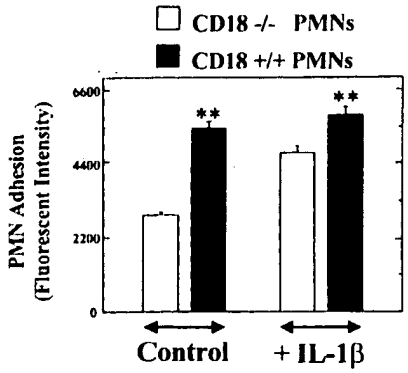

Figure 1. CD18-dependent binding of neutrophils to quiescent or activated endothelium. Murine brain endothelial cells[21] were grown to confluence on a 24-well plate and either left unstimulated (Control) or exposed to recombinant murine IL-1b for 20 hours. Neutrophils were harvested from either CD18 −/− or CD18 +/+ mice, fluorescently labeled, and coincubated with the endothelial cells. After a series of repetitive washes, adherent cells were detected based on the emission of 530 nm light in response to application of an excitation wavelength of 485 nm. The results shown represent the data from 5 endothelial cell monolayers; values shown are mean±SEM. $^{**}P<0.01$.

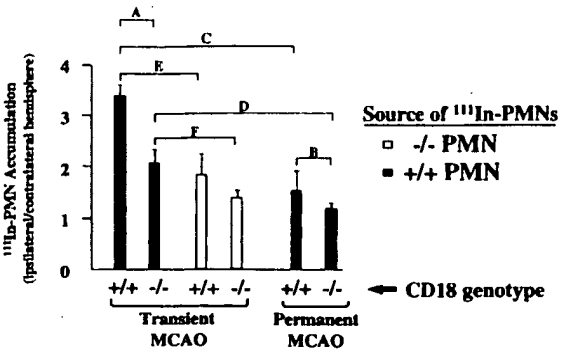

Figure 2. Adoptive transfer experiments demonstrating relative ^{111}Indium-labeled PMN accumulation in transient or permanent middle cerebral artery occlusion (MCAO). The abscissa denotes the groups of animals subjected to transient or permanent MCAO and the CD18 genotype of the animals undergoing occlusion. Filled bars represent animals injected with radiolabeled CD18 +/+ PMNs. Open bars represent animals injected with radiolabeled CD18 −/− PMNs. Comparison A, radiolabeled CD18 +/+ PMNs injected in CD18 +/+ (n=3) or CD18 −/− (n=6) animals under conditions of transient MCAO ($P=0.02$); comparison B, radiolabeled CD18 +/+ PMNs injected in CD18 +/+ (n=5) or CD18 −/− (n=5) animals under conditions of transient MCAO (P=NS); comparison C, radiolabeled CD18 +/+ PMNs injected into CD18 +/+ animals under conditions of transient (n=3) or permanent (n=5) MCAO ($P=0.02$); comparison D, radiolabeled CD18 +/+ PMNs injected into CD18 −/− animals under conditions of transient (n=6) or permanent (n=5) MCAO ($P=0.02$); comparison E, CD18 +/+ animals injected with radiolabeled CD18 +/+ PMNs (dark bar, n=3) or radiolabeled CD18 −/− PMNs (open bar, n=5) and subjected to transient MCAO ($P=0.046$); comparison F, CD18 −/− animals injected with radiolabeled CD18 +/+ PMNs (dark bar, n=6) or radiolabeled CD18 −/− PMNs (open bar, n=6) and subjected to transient MCAO ($P=0.057$).

anterior, posterior, and right/left circulations (Data not shown).

Because CD18 is expressed on the neutrophil surface, in contrast to its counterligand ICAM-1, which is an integral endothelial membrane protein, the next set of experiments used an adoptive transfer strategy in which either radiolabeled CD18 +/+ or CD18 −/− PMNs could be infused into either CD18 +/+ or CD18 −/− experimental animals subjected to transient MCA occlusion. Initial experiments were performed to demonstrate that CD18 −/− neutrophils do indeed exhibit diminished capacity to bind to both quiescent endothelium, as well as endothelium which has been activated with IL-1, a known potent inducer of ICAM-1 on the endothelial cell surface.[24] These studies [Figure 1] show that primary neutrophils, obtained from CD18 −/− mice, have a diminished capacity to bind to murine brain endothelial cells[21] both under resting conditions and after endothelial cells have been stimulated with IL-1.

To elucidate the role of CD18 in stroke, radiolabeled PMNs were infused into mice immediately prior to stroke, and their relative accumulation into the ischemic hemisphere quantified. The MCA of CD18 +/+ and CD18 −/− mice was transiently occluded (for 45 minutes) and then allowed to reperfuse for the duration of the 24 hour observation period. There was a significant accumulation of radiolabeled CD18 +/+ PMNs in the ipsilateral hemisphere of both CD18 +/+ and CD18 −/− mice (ratio>1.0). However, accumulation was greater in the CD18 +/+ mice [Figure 2, comparison A]. In contrast, when the MCA occluding suture was left in place to create a permanent model of stroke, at the same 24 hour time point, there was less overall PMN accumulation and no significant difference in relative ^{111}In-CD18 +/+ PMN accumulation in the ipsilateral hemisphere between CD18 +/+ and CD18 −/− mice [Figure 2, comparison B]. To assess the effect of different ischemic conditions, mice were subjected to either transient or permanent MCA occlusion (Figure 2, comparisons C & D). These data show that transient MCA occlusion is associated with greater ipsilateral PMN accumulation than following permanent MCA occlusion. Taken together, these data lead us to conclude that (1) CD18 −/− mice demonstrate reduced accumulation of PMNs compared with CD18 +/+ animals subjected to transient ischemia, and (2) transient cerebral ischemia with its associated reperfusion causes a greater accumulation of neutrophils in the ischemic hemisphere than under conditions of permanent MCA occlusion.

These experiments, in which CD18-expressing PMNs accumulated to a lesser degree in CD18 −/− than CD18 +/+ animals [Figure 2, comparison A], suggested to us that there is a role for neutrophil-induced neutrophil recruitment; the defect in CD18 expression on native neutrophils in CD18 −/− animals would not otherwise be expected to alter the recruitment of CD18 +/+ neutrophils to the ischemic cerebral microvasculature, because the CD18 −/− animals are not deficient for the CD18 counterligand, ICAM-1, or other adhesion receptors. Initial experiments using labeled CD18 −/− PMNs showed reduced accumulation compared with labeled CD18 +/+ PMNs [Figure 2, comparisons E & F], confirming the relative importance of CD18 expression in PMN recruitment in stroke (as was shown in comparison A). To examine the relative importance of non–CD18-dependent

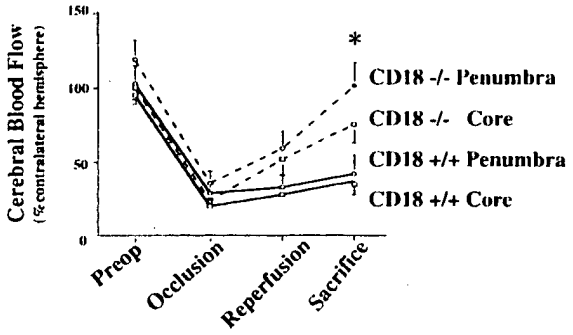

Figure 3. Effect of CD18 expression on relative CBF in mice subjected to transient MCA occlusion. Preop indicates prior to occlusion of MCA; Occlusion, approximately 1 minute after occlusive catheter introduced into the ICA; Reperfusion, approximately 1 minute after the occlusive catheter was removed; and Sacrifice, 24 hours after transient MCA occlusion. *P=0.02 between relative CBF in penumbra of CD18 −/− mice (dashed line, n=14) compared with CD18 +/+ (solid line, n=26) mice.

mechanisms of neutrophil recruitment, we next performed adoptive transfer experiments using radiolabeled CD18 −/− PMNs. Because these CD18-deficient PMNs provide the label (and therefore, are the only cells whose accumulation is tracked), the most likely explanation for their recruitment to ischemic foci is their adhesion via non–CD18-dependent mechanisms. When CD18 −/− PMNs were infused just prior to transient MCA occlusion [Figure 2, open bars], the relative accumulation of neutrophils in the ipsilateral compared with the contralateral hemisphere exceeded unity in both CD18 −/− and CD18 +/+ mice, suggesting the participation of non–CD18-dependent adhesive mechanisms (eg, selectins) in the capture of neutrophils.

Stroke Outcome

The next series of experiments was designed to study the functional significance of CD18 expression in stroke. For these experiments, measurements were made of CBF as well as infarct volumes in both CD18 +/+ and CD18 −/− mice. Preoperative relative CBFs, measured as the ratio of ipsilateral (right) to contralateral (left) hemispheric Doppler signals, was similar for both CD18 −/− and CD18 +/+ mice [Figure 3, "Preop"]. Both groups demonstrated reduction of blood flow which exceeded 50% at the time the suture was placed at the level of the MCA [Figure 3, "Occlusion"]. After 45 minutes of occlusion, the MCA occluding suture was withdrawn, the animal was turned prone, the Doppler probe was positioned, and relative CBF was recorded [Figure 3, "Reperfusion"]. Even at this relatively early time point, there was a tendency for relative CBF to be higher in the CD18 −/− animals than the CD18 +/+ controls. By the time of sacrifice at 24 hours, this difference became more pronounced and statistically significant in the penumbral region [Figure 3, "Sacrifice"].

To establish the overall pathophysiological significance of CD18 expression in stroke, cerebral infarct volumes were calculated. In transient cerebral ischemia, there was a marked (53%) reduction in cerebral infarct volumes in CD18 −/− mice compared with CD18 +/+ mice (P<0.05; Figure 4).

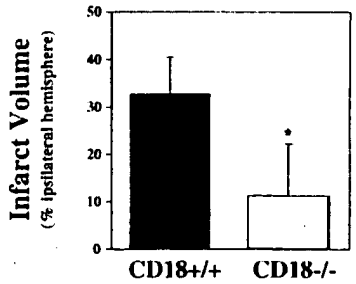

Figure 4. Effect of CD18 expression on cerebral infarct volume after transient MCA occlusion. Relative infarct volumes measured at death 24 hours after the ischemic event were quantified by TTC staining of serial cerebral sections, followed by computer-assisted planimetric analysis. Infarct volumes are expressed as percent ipsilateral hemisphere. Filled bars (n=14) represent infarct volumes in CD18 +/+ animals, and open bars (n=26) denote CD18 −/− mice. Values shown are mean±SEM; *P=0.04 between groups.

This reduction in infarct volumes in the CD18 −/− mice was accompanied by a reduction in mortality (3.7-fold reduction in mortality, P<0.02 versus CD18 +/+ mice). In contrast, when mice were subjected to permanent cerebral ischemia, no differences were noted in either infarct volumes or mortality between the two groups [Figure 5]. When mice were examined for neurological deficit (prior to anesthesia and sacrifice) at the 24-hour time point, there was a trend toward reduced neurological deficit in the CD18 null mice for both transient and permanent middle cerebral artery occlusion [Figure 6].

Discussion

Safe therapeutic options for the treatment of evolving stroke are extremely limited. Although reports by the NINDS[2] and ECASS[3] suggest that establishing early reperfusion with thrombolytic agents may help reduce the neurologic morbidity and mortality of a thrombotic or embolic stroke, the window of opportunity for therapeutic administration is exceedingly narrow. There are clearly theoretical advantages to establishing reperfusion (ie, restoration of oxygen and nutrient delivery to ischemic cerebral tissue), yet there is also the possibility that reintroduction of blood and blood-borne cells (such as PMNs) can exacerbate damage due to ischemia per se.[9] There are numerous mechanisms by which PMNs can

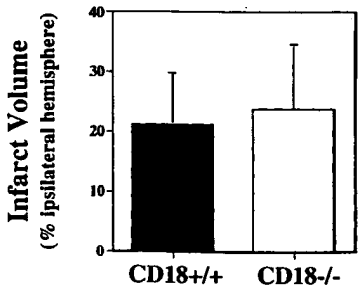

Figure 5. Effect of CD18 expression on cerebral infarct volume after permanent MCA occlusion. Filled bars (n=10) represent infarct volumes in CD18 +/+ animals, and open bars (n=12) denote CD18 −/− mice. Values shown are mean±SEM; P=NS between groups.

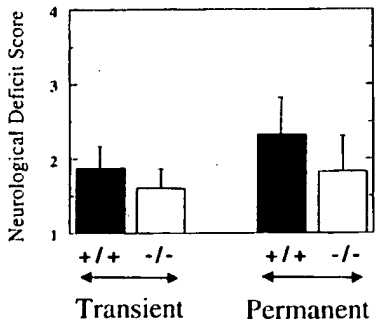

Figure 6. Effect of CD18 expression on neurological deficit after transient or permanent MCA occlusion. The +/+ or −/− symbols denote the CD18 genotype of the mice subjected to stroke. Before anesthesia and death at 24 hours, neurological deficit was scored according to a previously published scoring system,[18] which is detailed in "Materials and Methods" (4 represents the greatest neurological deficit). Animals used were the same as those used to calculate cerebral infarct volumes (Figures 4 and 5). Values shown are mean±SEM; P=NS between groups.

exacerbate cerebral damage following ischemia. As the ever-ready sentinels of the immune system, PMNs are laden with an arsenal of toxic and reactive species[7]. In addition, PMNs may potentiate microvascular occlusion by direct mechanical obstruction of flow, due to adhesion to microvascular endothelium and by stiffening on activation. In fact, activated PMNs rapidly upregulate surface CD18 expression,[25,26] which can amplify their adhesive potential. Previous studies have confirmed both a detrimental role for neutrophils in the pathogenesis of cerebral tissue injury in stroke, as well as a contributory role for various leukocyte adhesion receptors, such as ICAM-1[11] and P-selectin.[10]

In the current work, we explored the pathogenic role of an important leukocyte counterligand to endothelial ICAM-1 (CD18) in stroke. CD18 is a member of the integrin superfamily of adhesion glycoproteins, a family which consists of a number of membrane spanning glycoproteins that promote cell-cell and cell-matrix interactions. Integrins are heterodimers consisting of 1 unique alpha subunit and 1 of 3 common β-subunits: β_1, β_2, and β_3. Within the β_2 family, there exist 3 distinct heterodimers, with the common CD18 subunit shared by distinct subunits (CD11a, CD11b, and CD11c). The CD11b/CD18 heterodimer (Mac-1) avidly binds to ICAM-1 on the endothelial surface, as does CD11a/CD18 (LFA-1), although the latter also binds ICAM-2 and ICAM-3. Both bind fibrinogen, as does CD11c/CD18. Although mice that lack CD18 cannot show ICAM-1 dependent cellular adhesion mediated via either Mac-1 or LFA-1, there are additional adhesive effector mechanisms (such as P-selectin–mediated leukocyte adhesion[10]) which may still be active in CD18-null mice. The studies presented here show that CD18 −/− mice exhibit diminished leukocyte recruitment and are significantly protected in the setting of reperfused stroke, indicating a potent pathophysiological role for CD18 in this setting. However, somewhat unexpectedly, in the setting of permanent focal cerebral ischemia, the lack of CD18 was not protective. These data suggest that a leukocyte antiadhesive strategy may work best when combined with a reperfusion therapy. In support of this hypothesis, antibody to ICAM-1 enhanced the ability of tPA to improve neurological outcome in a rabbit model of embolic stroke, and antibody to CD18 showed efficacy with tPA at doses that were by themselves ineffective.[27]

The current studies also permitted us to examine the general proinflammatory role of CD18 in the postischemic brain. The topographic localization of CD18 on circulating leukocytes rather than as a fixed adhesion receptor on the vessel wall enabled us to perform adoptive transfer experiments, which demonstrated that neutrophil recruitment amplifies neutrophil recruitment. In the first of these experiments, we injected radiolabeled CD18 +/+ PMNs into CD18 +/+ or CD18 −/− mice subjected to transient focal cerebral ischemia under conditions identical to prior experimental protocols and compared these data to CD18 +/+ PMNs. Because other than hypomorphism for CD18, the counterligands and other adhesion receptors are functionally intact in both types of recipient mice, one would expect no significant difference in the amount of accumulation of radiolabeled CD18 +/+ PMNs between the 2 groups. However, this was not the case; there was a significantly diminished binding of these PMNs in CD18 −/− animals. As the preponderant population of native PMNs in each recipient presumably accumulates to a greater or lesser degree depending on the presence or absence of functional CD18, it is reasonable to speculate that reduced accumulation of the native PMN population in the CD18 −/− animals inhibited further recruitment of PMNS with fully competent adhesion receptors. Proof of reduced accumulation of CD18 −/− PMNs in the CD18 −/− mice comes from the adoptive transfer experiments in which CD18 −/− PMN deposition was tracked in CD18 −/− mice; these mice had the lowest PMN deposition of all groups of mice subjected to transient cerebral ischemia.

The current studies contribute to the growing evidence implicating a detrimental role for PMNs in stroke. In the mouse model of stroke, absolute reduction in the numbers of circulating PMNs before transient focal ischemia is by itself sufficient to improve stroke outcome.[11] Although leukocyte recruitment occurs within minutes of reperfusion in a murine model of stroke, it continues for at least the ensuing 24 hours and is only partially blocked by the absence of the P-selectin gene,[10] suggesting an active role for other mechanisms of leukocyte recruitment. In humans, CD11a and CD18 are both upregulated in the leukocytes of patients with ischemic stroke and transient ischemic attacks.[28] However, it has been difficult to tease out the pathogenic role of CD18 in PMN recruitment in stroke using blocking antibodies. Administration of a blocking antibody to CD18 (clone designate R 3.3) demonstrated therapeutic efficacy effective in an ischemia-reperfusion model of spinal cord injury[29] but not in a model of irreversible cerebral embolic stroke.[16] In the latter study, administration of a monoclonal antibody to CD18 (clone designate MoAb 60.3) did not improve CBF or evoked potentials. By using mice with severe functional hypomorphism of the CD18 gene product, the current studies support our hypothesis that CD18 is indeed pathogenic in PMN recruitment and cerebral tissue damage in stroke. Taken together, these studies demonstrate that reperfusion repre-

sents an especially vulnerable period for the brain, providing the potential benefits of restoring nutritive blood flow to an ischemic region while simultaneously opening the flood gates for a massive influx of activated PMNs. These studies suggest that stroke outcomes may be improved by anti-leukocyte adhesive strategies that are specifically targeted to the reperfusion period.

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References

1. Zivin JA, Fisher M, DeGirolami U, Hemenway CC, Stashak JA. Tissue plasminogen activator reduces neurological damage after cerebral embolism. *Science*. 1985;230:1289–1292.
2. The National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group. Tissue plasminogen activator for acute ischemic stroke. *N Engl J Med*. 1995;333:1581–1587.
3. Hacke W, Kaste M, Fieschi C, Toni D, Lesaffre E, von Kummer R, Boysen G, Bluhmki E, Hoxter G, Mahagne MH, Hennerici M, for the ECASS Study Group. Intravenous thrombolysis with recombinant tissue plasminogen activator for acute hemispheric stroke. *JAMA*. 1995;274: 1017–1025.
4. Gonner F, Remonda L, Mattle H, Sturzenegger M, Ozdoba C, Lovblad KO, Bassetti C, Schroth G. Local intra-arterial thrombolysis in acute ischemic stroke. *Stroke*. 1998;29:1894–1900.
5. del Zoppo GJ, Higashida RT, Furlan AJ, Pessin MS, Rowley HA, Gent M, and the PROACT Investigators. PROACT: a phase II randomized trial of recombinant pro-urokinase by direct arterial delivery in acute middle cerebral artery stroke. [see comments]. *Stroke*. 1998;29:4–11.
6. The Multicenter Acute Stroke Trial. Thrombolytic therapy with streptokinase in acute ischemic stroke. *N Engl J Med*. 1996;335:145–150.
7. Weiss SJ. Tissue destruction by neutrophils. *N Engl J Med*. 1989;320: 365–376.
8. Braunwald E, Kloner RA. Myocardial reperfusion: a double edged sword? *J Clin Invest*. 1985;76:1713–1719.
9. Aronowski J, Strong R, Grotta JC. Reperfusion injury: demonstration of brain damage produced by reperfusion after transient focal ischemia in rats. *J Cereb Blood Flow Metab*. 1997;17:1048–1056.
10. Connolly ES Jr, Winfree CJ, Prestigiacomo CJ, Kim SC, Choudhri TF, Hoh BL, Naka Y, Solomon RA, Pinsky DJ. Exacerbation of cerebral injury in mice which express the P-selectin gene: identification of P-selectin blockade as a new target for the treatment of stroke. *Circ Res*. 1997;81:304–310.
11. Connolly ES Jr, Winfree CJ, Springer TA, Naka Y, Liao H, Yan SD, Stern DM, Solomon RA, Gutierrez-Ramos J-C, Pinsky DJ. Cerebral protection in homozygous null ICAM-1 mice after middle cerebral artery occlusion. Role of neutrophil adhesion in the pathogenesis of stroke. *J Clin Invest*. 1996;97:209–216.
12. Polmar SH, Sherman DS. Double-blind, randomized, placebo-controlled, parallel-group trial of the efficacy and safety of enlimomab (anti-ICAM-1) compared to placebo administered within 6 hours of the onset of symptoms for the treatment of acute ischemic stroke. *22nd International Joint Conference on Stroke and Cerebral Circulation (Abstract CT-10)* 1996.
13. Springer TA. Adhesion receptors of the immune system. *Nature*. 1990; 346:425–434.
14. Matsuo Y, Onodera H, Shiga Y, Shozuhara H, Ninomiya M, Kihara T, Tamatani T, Miyasaka M, Kogure K. Role of cell adhesion molecules in brain injury after transient middle cerebral artery occlusion in the rat. *Brain Res*. 1994;656:344–352.
15. Lindsberg PJ, Siren AL, Feuerstein GZ, Hallenbeck JM. Antagonism of neutrophil adherence in the deteriorating stroke model in rabbits. *J Neurosurg*. 1995;82:269–277.
16. Takeshima R, Kirsch JR, Koehler RC, Gomoll AW, Traystman RJ. Monoclonal leukocyte antibody does not decrease the injury of transient focal cerebral ischemia in cats. *Stroke*. 1992;23:247–252.
17. Wilson RW, Ballantyne CM, Smith CW, Montgomery C, Bradley A, O'Brien WE, Beaudet AL. Gene targeting yields a CD18-mutant mouse for study of inflammation. *J Immunol*. 1993;151:1571–1578.
18. Connolly ES Jr, Winfree CJ, Stern DM, Solomon RA, Pinsky DJ. Procedural and strain-related variables significantly affect outcome in a murine model of focal cerebral ischemia. *Neurosurgery*. 1996;38: 523–532.
19. Bederson JB, Pitts LH, Nishimura MC, Davis RL, Bartkowski HM. Evaluation of 2,3,5-triphenyltetrazolium chloride as a stain for detection and quantification of experimental cerebral infarction in rats. *Stroke*. 1986;17:1304–1308.
20. Akeson AL, Woods CW. A fluorometric assay for the quantitation of cell adherence to endothelial cells. *J Immunol Methods*. 1993;163:181–185.
21. Gumkowski F, Kaminska G, Kaminski M, Morrissey LW, Auerbach R. Heterogeneity of mouse vascular endothelium: in vitro studies of lymphatic, large blood vessel and microvascular endothelial cells. *Blood Vessels*. 1987;24:11–23.
22. Yan S-F, Tritto I, Pinsky DJ, Liao H, May L, Stern DM. Induction of interleukin 6 (IL-6) by hypoxia in vascular cells: central role of the binding site for nuclear factor-IL-6. *J Biol Chem*. 1995;270: 11463–11471.
23. Barone FC, Knudsen DJ, Nelson AH, Feuerstein GZ, Willette RN. Mouse strain differences in susceptibility to cerebral ischemia are related to cerebral vascular anatomy. *J Cereb Blood Flow Metab*. 1993;13: 683–692.
24. Pober J. Warner-Lambert Parke Davis Award Lecture: cytokine-mediated activation of vascular endothelium. *Am J Pathol*. 1988;133:426–433.
25. Naccache PH, Jean N, Liao NW, Bator JM, McColl SR, Kubes P. Regulation of stimulated integrin surface expression in human neutrophils by tyrosine phosphorylation. *Blood*. 1994;84:616–624.
26. Sengelov H, Kjeldsen L, Diamond MS, Springer TA, Borregaard N. Subcellular localization and dynamics of Mac-1 (alpha m beta 2) in human neutrophils. *J Clin Invest*. 1993;92:1467–1476.
27. Bowes MP, Rothlein R, Fagan SC, Zivin JA. Monoclonal antibodies prevent leukocyte activation, reduce experimental neurological injury, and enhance efficacy of thrombolytic therapy. *Neurology*. 1995;45: 815–819.
28. Kim JS, Chopp M, Chen H, Levine SR, Carey JL, Welch KM. Adhesive glycoproteins CD11a and CD18 are upregulated in the leukocytes from patients with ischemic stroke and transient ischemic attacks. *J Neurol Sci*. 1995;128:45–50.
29. Clark WM, Madden KP, Rothlein R, Zivin JA. Reduction of central nervous system ischemic injury in rabbits using leukocyte adhesion antibody treatment. *Stroke*. 1991;22:877–883.

Editorial Comment

The study by Prestigiacomo et al provides evidence that the adhesion receptor CD18, which is a key receptor for neutrophil binding to activated endothelium, is important in neutrophil mediation of cerebral injury following ischemia and reperfusion. These data were contrasted with lack of involvement of the CD18 adhesion receptor and neutrophils in ischemic injury induced by permananent occlusion of the middle cerebral artery. Assuming that the model used for the study (ie, genetic manipulations that lead to deletion of the gene that expresses the CD18 receptor) does not encompass

aberrations beyond the expected (for example, significant changes in cerebral microvessels, circulation, and tissue due to "lifelong" diminished encounter with neutrophils), the study points to the possibility that antiadhesion strategies aimed at disruption of the interaction of neutrophils with the endothelium could result in histological and functional benefits only in cases in which reperfusion of the ischemic zone has been established after a brief (45-minute) ischemic period. The clinical significance of this observation needs to be evaluated vis-à-vis the clinical reality as follows: (1) Is reperfusion of ischemic brain in the common stroke situation a prevalent phenomenon? (What percentage of the patients with MCA occlusion reperfuse?) This issue has significant literature background to suggest that, indeed, a significant number of patients reperfuse (30% to 70%).[1-4] (2) Does reperfusion in stroke patients occur early enough and in a significant number of patients to afford therapeutic opportunity? In fact, the majority of patients who reperfuse are not within the early (few hours) time frame[5]; late reperfusion (12 to 24 hours to week[s]) may not provide/result in medical benefits because (a) the erratic nature of the event (no predictors) and (b) the reperfusion may not be of "nurturing quality," as the tissue perfused may be largely dysfunctional at that time. Furthermore, it is not clear whether agents that work in models of reperfusion demonstrate efficacy because their action needs the presence of blood flow or because of reperfusion injury which the agent abolishes. Thus, it is highly questionable whether therapeutic agents aimed to block the CD18 adhesion receptors will be useful therapy in the majority of stroke patient treated within few hours after the ictus; in fact, the recent failure to establish efficacy with enlimomab (the anti–ICAM-1 murine antibody) in stroke trials supports this suggestion (with the caveats that the latter agent's liabilities and mode of delivery and not the mechanism of action are at fault). In any case, anti-CD18 agents may be useful as adjunct therapeutics to targeted and timed reperfusion induced by tPA (and other thrombolytics), either by systemic or local delivery, at a certain time frame that is as yet unspecified in humans.

From the preclinical research perspective, it is rather intriguing to speculate on reasons that CD18-deficient mice had no improved outcome after permanent ischemia. Current dogma supports a role for the penumbra region in the outcome of the ischemic damage. Because the penumbra is perfused, albeit at lower level, neutrophil accumulation and its putative consequence—exacerbation of damage—should have been noticed. Alas, this elegant study is a perfect example of how preclinical data may provide compelling evidence for a potential therapeutic utility of an available agents (anti-CD18–neutralizing antibodies), yet careful analysis of the clinical context must be exercised to identify the discrete opportunity, if any, of CD18 antagonists in stroke.

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References

1. Solis OJ, Robertson GR, Taveras JM, Mohr J, Pessin MS. Cerebral angiography in acute cerebral infarction. *Rev Interam Radiol.* 1997;2: 19–25.
2. Irino T, Taneda M, Minami T. Angiographic manifestations in post-recanalized cerebral infarction. *Neurology.* 1997;27:471–475.
3. Fieschi C, Argentino C, Lenzi GL, Sacchetti ML, Toni D, Bozzao L. Clinical and instrumental evaluation of patients with ischemic stroke within the first six hours. *J Neurol Sci.* 1989;91:311–321.
4. del Zoppo GJ, Poeck K, Ressin MS, Wolpert SM, Furlan AJ, Ferbert A, Alberts MJ, Zivin JA, Wechsler L, Busse O, Greenlee R Jr, Brass L, Mohr JP, Feldmann E, Hacke W, Kase CS, Biller J, Gress D, Otis SM. Recombinant tissue plasminogen activator in acute thrombotic and embolic stroke. *Ann Neurol.* 1992;32:78–86.
5. del Zoppo G, Higashida RT, Furlan AJ, Pessin MS, Rowley HA, Gent M, and the PROACT Investigators. PROACT: A phase II randomized trial of recombinant prourokinase by direct arterial delivery in acute middle cerebral artery storke. *Stroke.* 1998;29:4–11.